Adenovirus 36 Seropositivity is Strongly Associated With Race and Gender, But Not Obesity, Among U.S. Military Personnel

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ORIGINAL ARTICLE

Adenovirus 36 seropositivity is strongly associated with race and gender, but not obesity, among US military personnel

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Background: Although several studies have shown a positive association between evidence of anti-adenovirus 36 (Ad-36) antibodies (Ad-36 exposure) and (1) obesity and (2) serum cholesterol in animals, there is limited research demonstrating this association in humans. There is also limited research on transmission, presentation and demographics of Ad-36 infection.
Design: (1) Body mass (body mass index (BMI)), (2) fasting serum cholesterol and triglyceride levels and (3) demographic characteristics were compared between Ad-36 seropositive and seronegative groups. The majority of subjects were matched as cases versus controls on a number of demographic variables.
Subjects: A total of 150 obese and 150 lean active-duty military personnel were studied.
Measurements: Subjects completed a questionnaire regarding demographic and behavioral characteristics. Subject serum samples were tested by serum neutralization assay for the presence of anti-Ad-36 antibodies.
Results: In all, 34% of obese and 39% of lean subjects had Ad-36 exposure, an insignificant difference. Serum cholesterol and triglyceride levels were significantly higher among the obese subjects than among the lean, but there were no associations between serum cholesterol and triglyceride levels and Ad-36 exposure. Positive associations were found between Ad-36 exposure and age, race and gender.
Conclusion: The study stands in contrast to previous work that has shown a positive relationship between Ad-36 exposure and (1) obesity, and (2) levels of serum cholesterol and triglycerides. In this study there was no association in either case. Unanticipated relationships between Ad-36 exposure and age, race and gender were found, and this is the first time that such a link between Ad-36 exposure and demographics has been found.
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Introduction

The traditional belief that obesity is a result of the cardinal sins 'gluttony' and 'sloth' came to be modified by the well-known formula that weight loss could be achieved by expending more calories than consuming each day, with the common presumption that expending calories was primarily a function of exercising. Over the past 20 years it has become clear that both the metabolic and extra-metabolic processes involved in expending and storing calories are varied and complex. Two people of similar size can in fact intake and expend by exercise identical amounts of energy each day and yet differ significantly in their percentage of body fat.[1]

Although eating and exercise behaviors are probably the primary factors, a more complete picture of the factors involved in the development of obesity has led to investigations of several categories of variations among individuals,[2] including social (for example, Christakis *et al.*[3]), genetic (for example, Bouchard *et al.*[4]) and hormonal (for example, Camilleri *et al.*[5]) factors, as well effects of gastric microbial flora (for example, Backhed *et al.*[6]). In addition, a line of research led by Dhurandhar *et al.*[11] has argued that viral and bacterial infections may have an important role.[7–9] In 1992, they found that, compared with controls, chickens

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inoculated with SMAM-1—an avian adenovirus—developed significantly greater adiposity, and paradoxically lower levels of serum cholesterol and triglycerides, even though the two groups had identical portions of food and, presumably, though not reported, amounts of exercise.[10]

A significant development in this story was introduced when a subsequent study on obese humans found that those positive for anti-SMAM-1 neutralizing antibodies had a significantly higher body mass index (BMI) than those who were seronegative.[7] This was surprising, as it was not known that humans were susceptible to avian adenoviruses. It should be noted that the direct connection between SMAM-1 infection and positive serological titers for SMAM-1 in humans has not been demonstrated, and could conceivably represent cross-reactive antibodies raised in response to human adenoviruses or other antigenic entities. It was also found, counter intuitively, that antibody-positive obese subjects had 15% lower serum cholesterol and 60% lower serum triglycerides than the non-obese subjects.

Parallel to these findings was a demonstration that adiposity could be induced in chickens and mice by infecting them with a human adenovirus-36 (Ad-36).[11] Further research showing such a relationship in animals included studies on rats,[12] and marmosets and rhesus monkeys.[13] Demonstrating the relationship in humans by experimental inoculation of Ad-36 would be unethical, and natural human infections with Ad-36 are rarely identified, so indirect methodologies have been employed to examine the relationship between previous infection with Ad-36, based on the presence of anti-Ad-36 neutralizing antibodies, and some measure of the individual's present weight or body-fat composition.

A study on humans using serology to determine previous exposure to Ad-36,[14] which extended the findings reported in two earlier articles,[15,16] found that 30% of the obese ($BMI > 29\,kg\,m^{-2}$) subjects were antibody-positive compared with 11% of the non-obese ($BMI \leqslant 29\,kg\,m^{-2}$) subjects. As in the previous studies on animals, levels of cholesterol and triglycerides were significantly higher in the antibody-negative subjects than the antibody positive. Similar results were found in twin pairs discordant for Ad-36 antibodies.[14]

An alternative methodology, reported in an unpublished study presented at a scientific conference,[17] was to infect human adipose-derived stem cells with Ad-36. This resulted in differentiation to adipocytes and lipid accumulation. The authors argued that Ad-36 inducement of adipogenesis in stem cells *in vitro* suggests that a similar process may occur *in vivo*.

The identification of factors related to body composition is of crucial importance to society at large. The prevalence of obesity in the United States of America has increased from 13 to 32% between 1960 and 2004.[18] In the military, where weight and fitness standards are imposed in the interest of health and performance of personnel, this is a particularly acute problem.

In this study, body mass, serum cholesterol and triglyceride levels, evidence of anti-Ad-36 antibodies, and demographic characteristics were obtained from both obese and lean military personnels who gave blood samples and completed a questionnaire. Of primary interest was a comparison of the preponderance of Ad-36 seropositivity in each of the body-mass groups, as well as the association of Ad-36 seropositivity with cholesterol and triglycerides. Relationships between various demographic variables and seropositivity of Ad-36 were also explored to understand the background of this poorly studied pathogen. We hypothesized that the study would support previously reported findings.

Subjects and methods

Subjects were active-duty US military personnel. Based on the prevalence of Ad-36 antibodies found in a previous study[16] —30% among obese subjects and 5% among non-obese subjects—300 subjects, 50% obese and 50% non-obese, were recruited.

All subjects gave two 8.5 ml serum-separator tubes of blood after a 12-h fast (defined as no ingestion of solid or liquid foods, except water). They also completed a demographic–weight–medical-history questionnaire that included several items. Basic demographic questions included gender, age and race. Questions on medical conditions included history of diabetes, hypothyroidism, other endocrine disorders, polycystic ovary syndrome, smoking habits and pregnancy, and whether the subject had previously received an adenovirus vaccination. Questions on diet and weight included eating habits (types of foods eaten and frequency), and reported weight gain (10–20 lb weight gains in the past year and their correlation with changes in physical activity and diet). Questions on physical condition included significant present and past injuries and activity level (frequency, duration and intensity of exercise each week, type of exercise and time on the military physical fitness running/swimming test). All subjects were paid $50.

A case was defined as a regular active-duty military member stationed in California who met the World Health Organization (WHO) and Center for Disease Control (CDC) BMI criteria for obesity. By these criteria, an individual with a BMI >29 is defined as obese. Frankenfield *et al*.[19] argued that such a criterion can confidently be presumed, although they found that a significant proportion of people whose BMI was <29 were also technically obese. Controls were defined as subjects who met the WHO/CDC criteria for absence of weight problems, that is, a BMI <26, and had no history (as evidenced by the questionnaire) of serious weight-control problems. Women who were pregnant and up to 6 months post partum were ineligible for the study.

Case-control matching on gender, age, race/ethnicity and military pay grade was achieved for two-thirds of the subjects. There were 4 levels for age (17–21, 22–25, 26–31 and $\geqslant 32$ years), 4 levels for race/ethnicity (white, black, Hispanic Latino and Asian) and 3 levels for pay grade (E1–E5, E6–E8 and O1–O5).

Laboratory procedures

Laboratory personnel were blinded to the case–control status associated with each specimen. Cholesterol and triglyceride panels were run on the study sera. Total serum cholesterol (LDL and HDL) was determined by phosphate-peroxidase assay using an Ortho Diagnostics (Johnson & Johnson Clinical Diagnostics, Rochester, NY, USA) Vitros 950 analyzer with dry slides. Absorbance was read at 540 nm. Acceptable values were set at <200 mg/dl. Cholesterol levels between 200–239 mg/dl were considered borderline, whereas values greater than 240 mg/dl were considered high. For triglycerides, absorbance was also read at 540 nm. Acceptable range was 30–190 mg/dl.

To test for the presence of neutralizing antibodies for Ad-36, our laboratory used the serum neutralization test similar to that described by Dhurandhar *et al*.[11] with the addition of colorimetric reading of microtiter plates at the end of the incubation period using a spectrophotometer. The plates were stained using neutral red solution after the incubation period and read using a Biotek Instruments (Winooski, VT, USA) ELx808 microplate reader at 550-nm wavelength. A positive titer was defined as a ratio of >1:4. Serum samples were heat-inactivated at 56 °C for 1 h and then diluted twofold from 1:4 to 1:512. Equal volumes of serum dilutions and virus suspension containing 100 $TCID_{50}$ Ad-36 were mixed in designated wells and then incubated for 1 h at 37 °C, 5% CO_2. After incubation, 5000 A-549 cells were added to all the wells.

Each serum dilution was assayed in duplicate wells. Negative control serum known to be negative for Ad-36 antibodies and positive control serum consisting of Ad-36 antibodies were included in each run. After the virus–serum dilution mixture was added, 1 h of incubation allowed antibodies (if present) to interact/neutralize the Ad-36 virus. After initial incubation, cells were added and were incubated for 7–10 days and observed for the development of characteristic adenovirus cytopathic effect. Positive neutralization was defined as the highest serum dilution that exhibited absorbance values equal to or greater than that of the control wells.

Forty-four of the samples were sent to Dr Dhurandhar's laboratory at the Wayne State University, Detroit, MI, USA where they were assayed as previously described.[7]

Data analysis

Statistical comparison of fasting cholesterol and triglyceride levels were made between cases and controls. General linear modeling was used to explore differences, within the obese group and within the lean group, between Ad-36 seropositive subjects and Ad-36 seronegative subjects with respect to triglyceride and lipid levels. Similarly, we evaluated within both obese and lean groups any difference in mean BMI between Ad-36 seropositive and Ad-36 seronegative subjects. The obese and lean subjects were also pooled to investigate whether there were differences in lipid levels between Ad-36 positive and Ad-36 negative subjects. Univariate modeling was used to evaluate the association between demographic components, such as age, gender, race and lifestyle variables such as diet and exercise, with being overweight and Ad-36 positivity. Additional covariates considered in the modeling included history of injuries, exercise habits, deployment history, medical conditions predisposing one to obesity, smoking, alcohol intake and family history.

Logistic regression models were built for each group of characteristics, such as diet, physical activity and blood measures to explore the interaction of multiple variables while simultaneously controlling for demographic differences between groups.

Statement of ethics

The study was approved by the Naval Health Research Center IRB and all subjects gave informed consent. We certify that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed during this research.

Results

Of the 293 valid subjects, there were 147 controls (lean by BMI) and 146 cases (obese by BMI). Of the 147 controls (lean by BMI), 57 (38.8%) were seropositive (titer >1:4) for presence of anti-Ad-36 antibodies (Table 1). Of the 146 cases (obese), 50 (34.3%) cases were positive. The continuous distribution of BMI over the seropositive individuals as a group versus the seronegative individuals as a group is shown in Figure 1. Each BMI is shown associated with the percent of the group having that BMI. Our obese subjects fell within a BMI range covering obesity class I (81% of our obese subjects), class II (17%) and class III (2%).

χ^2 analysis indicated a significant association between Ad-36 positivity and race (*P*-value 0.0075), female gender (*P*-value 0.036), and a lower frequency of high levels of low-density lipoproteins (*P*-value 0.013).

Logistic regression indicated no significantly different risk of obesity associated with antibody evidence of Ad-36 exposure. Geometric mean titers were 3.2 for the obese and 3.62 for the lean subjects. Figure 2 is a plot of numbers of subjects in each group against their respective titers, the curves for which reflect the lack of difference between the two groups. However, people over 26-years old were significantly less likely to have a history of Ad-36 when compared with people younger than 26 (age 26–31 years OR: 0.43, 95% CI 0.21–0.89; age >31 years OR: 0.38, 95% CI: 0.17–0.85). In addition, African–Americans were significantly more likely to have antibody evidence of Ad-36 exposure (OR: 2.65, 95% CI 1.37–5.15), and men overall were less likely than women to have similar evidence (OR: 0.59, 95% CI 0.34–1.01) (Table 1).

Table 1 Associations of body profile and adenovirus 36 (Ad-36) status, age and race/ethnicity

Level of factor	*Number of subjects Ad-36 positive*	*Percentage of all subjects Ad-36 positive*	*Number of subjects Ad-36 negative*	*Percentage of all subjects Ad-36 negative*	*Adjusted OR of Ad-36 positive*	*95% CI*
Body mass index (BMI)						
<26	57	53.3	90	48.4	1.00	
>29	50	46.7	96	51.6	1.07	0.61–1.87
Age (years)						
17–21	33	31	31	17	1.00	
22–25	25	23	41	22	0.54	0.26–1.12
26–31	29	27	65	35	0.43	0.21–0.89
>31	20	19	49	26	0.38	0.17–0.85
Race/ethnicity						
Caucasian	49	46	119	64	1.00	
Hispanic	28	17	24	13	1.89	0.93–3.87
African–American	18	26	25	13	2.65	1.37–5.15
Asian	11	10	12	7	2.01	0.81–5.01
Other	1	1	6	3	0.27	0.03–2.48
Gender						
Female	41	38	47	25	1.00	
Male	66	62	139	75	0.59	0.34–1.01

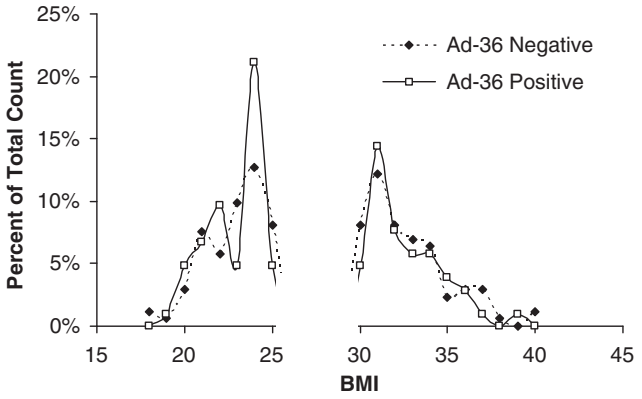

Figure 1 Distribution for lean (body nass index (BMI) <26) and obese (BMI >29) groups showing the percent of all subjects in the group who were positive or negative at each BMI.

Concordance was calculated on the specimens analyzed by both our laboratory and Dhurandhar's laboratory. Using only samples for which we have valid results (35 of the 44 sent to Dhurandhar's laboratory), and Dhurandhar's laboratory results as the gold standard, sensitivity was 100% and specificity was 76%. It was not possible to use Dhurandhar's results to analyze obesity and leanness against Ad-36 infection in the 44 samples, as all but one of them were from obese subjects.

After adjusting for other demographic variables, the association between Ad-36 positivity and lower rates of high LDLs was not found to be statistically significant. There was no significant difference in a 2×2 chi-square (P-value 0.4208) comparing the matched obese and lean subjects against a comparison of all obese to non-obese subjects.

χ^2 univariate analysis found significant associations between higher BMI and older age groups (P-value 0.002), African–American race and Hispanic ethnicity (P-value 0.01), male gender (P-value 0.015), and never having deployed or having spent a smaller proportion of military career deployed (P-value 0.016). In addition, higher BMI was significantly associated with variables characterizing a lower capacity for physical exertion (characterized by fewer pushups, fewer sit-ups, slower times on the physical fitness test and a higher frequency in reporting 'always sweating' during physical activities), reported weight gain, and diet and blood measures consistent with previous studies.

Logistic regression found a significant association between age and BMI, with each consecutive age category associated with an increased risk of obesity when compared with participants aged 17–21 years (age 22–25 years OR: 2.963, 95% CI: 1.3–6.7; age 26–31 years OR: 7.432, 95% CI: 3.9–16.3; age >31 years OR: 9.25 95% CI: 3.99–21.4). African–American and Hispanic participants had a significantly increased risk of obesity when compared with white participants (African–American OR: 2.3, 95% CI: 1.15–4.8; Hispanic OR: 2.1 95% CI: 1.0–4.6). Males were also found to have a significantly higher risk of obesity when compared with females (male OR: 1.8, 95% CI: 1.0–3.2).

As expected, higher capacity for physical exertion was associated with significantly lower risk of obesity. The ability to perform 70 or more pushups was associated with lower risk when compared with persons able to only perform 40 or less, as was the ability to perform 80 or more sit ups when compared with those who could only perform 59 or less. In addition, small amounts of alcohol consumption per month was associated with an increased risk, whereas moderate to

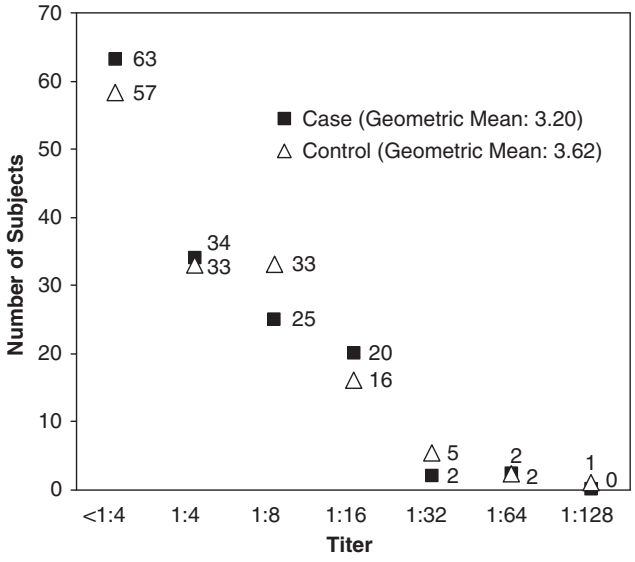

Figure 2 Number of subjects in each group at each titer. The curves of the groups are very similar, as are their geometric mean titers, reflecting the logistic regression showing no difference between them.

frequent drinking showed no difference in risk of obesity when compared with no alcohol consumption. Lower levels of HDLs and LDLs and higher levels of VLDLs and triglycerides were associated with an increased risk of obesity.

The mean cholesterol level for obese cases, 195.8 mg/dl, was significantly greater than the mean of 172.8 mg/dl for lean controls ($P<0.001$). The mean triglyceride level for obese cases was 143.2 mg/dl, which is significantly greater than the mean of 85.5 mg/dl for lean controls ($P<0.001$).

No significant difference was found within either the obese group or lean group when comparing Ad-36 seropositive and Ad-36 seronegative subjects with respect to mean BMI, triglyceride, or lipid levels. Neither did we find a significant difference between obese and lean subjects with respect to lipid levels when the Ad-36 positive and Ad-36 negative subjects were pooled.

Discussion

We found no difference between subjects who were obese (BMI >29) and those who were lean (BMI <26) with respect to evidence of previous Ad-36 infection. This is in contrast with previous research by Dhurandhar's group on human subjects[7,20] and animals.[10,21] As our study was motivated by an interest in expanding on these findings, in so far as it does not support them, their discussion requires particular attention to variables that may explain the discrepancies. We did, however, find strong associations of previous Ad-36 infection with (1) age, (2) race and (3) gender.

Although there is copious literature on transmissibility of other types of adenoviruses responsible for gastrointestinal, respiratory diseases (for example, Russell *et al*.[22]) and keratoconjunctivitis (for example, Dominguez-Berjon *et al*.[23]), there is little data on epidemiology, characteristics, and demographics of Ad-36 infection specifically. It is unclear what to make of the fact that people more than 25-years old were significantly less likely to have anti-Ad-36 antibodies. This could result from a recent increase in exposures among children and young adults, or it could result from waning immunity with advancing age (though this latter explanation cannot be confirmed by our data: there is no correlation between age and titer).

Findings related to race are, of course, difficult to interpret unless cultural differences can be eliminated as confounders. We could do no more than speculate as to why African–Americans have higher rates than the other groups examined. Our finding of a trend for women to have higher rates (nearly significant with OR 0.59, 95% CI 0.34–1.01) than men is also curious and difficult to explain. These are, in any case, the first findings of an association of Ad-36 with age, race or gender. With each of these factors, it may be worth keeping in mind that our population was military personnel, and it may be that there are interactions between age and military, race and military, and gender and military, as factors, that might explain our results.

On the question of obesity–adenovirus associations, it should be emphasized that a number of factors are associated with obesity, of which viral infections may be just one. Various characteristics of our study population could mitigate factors such as viral exposure. These might include the influence of military demands on fitness and BMI control among active-duty military personnel. In general, it might be reasonably assumed that the military population has a lower rate of obesity than the general population, engages in more physical activity, and is in better general health. The best available evidence suggests that obesity rates in the general US population are about twice that of the military population.[24] More physical activity among a population is thought to reduce rates of obesity,[25] and the best estimates suggest that military personnel engage in more physical activity than does the general population by a factor of 2 or 3 (cf[26]).

Such factors, should, however, be irrelevant as we were simply comparing matched obese and control populations, assuming that obese military personnel are like obese members of the general population in terms of exercise and fitness. However, this is probably not the case. The military obese population engages at far higher rates of 'regular' physical exercise than the general US population (cf[27] and Table 1). This would suggest that the obesity present in the military population has not been overcome by exercise, and that in fact our study population is obese *in spite of* regular exercise, which should increase the likelihood that their obesity is due to some other factor. Thus, evidence of more exercise in the military, and especially among the obese, instead of mitigating the effect of some factor such as viral exposure, increases its likelihood,

assuming that exercise affects different causes of obesity equivalently. It is significant that we did not find that previous infection with Ad-36 was such a factor.

As the rate of Ad-36 exposure is higher in our subjects than was reported in Atkinson *et al*'s[14] study on 502 subjects from various parts of the US, wherein they found approximately 27% to have Ad-36 antibodies, it would be reasonable to wonder whether the military environment is conducive to elevated transmission of Ad-36. Unfortunately, we have no direct evidence of this. In any case, we would still expect a higher proportion of the obese subjects to be Ad-36 positive than the non-obese subjects.

Apart from our results, it may be useful to consider the virus hypothesis with respect to the steady temporal increase in obesity rates in the US. Atkinson points to a temporal connection between the discovery of Ad-36[28] and the initiation of the world-wide epidemic of obesity.[20] This may be worth pursuing to determine whether Ad-36 infection rates have increased during the epidemic period. If they have not, then although they could partially explain endemic obesity, they would not explain the epidemic. If they have, then this could turn out to be a significant contribution not only to explain the cause of obesity but of the epidemic.

It is possible, on the other hand, that our laboratory results are confounded by some particular characteristic of our population having been at some point infected with another type of adenovirus, whose antibodies cross-reacted with the antigen of the assay. Compared with the general population, our population is known to be highly immune to Ad-4 and to have a relatively high degree of immunity to Ad-7.[22] However, although theoretically possible, Ad-4 (species E) and Ad-7 (species B) are members of different species from Ad-36 (species D), which might lead one to believe that cross-reactivity is less likely than if they were of the same species. This is not to say that cross-reactivity between species is not worth consideration. Ad4 and Ad7, for example, cross-react despite their being from different species.[22] Nevertheless, there is no evidence that these cross-react with Ad-36.

One possible limitation to the study's conduct was the misidentification of obese subjects. Given that military personnel do more physical exercise than people in the general population, it is perhaps easier to find military personnel whose BMI belies their actual body composition. Although BMI is a relatively stable estimate in general, if there were a strong bias toward misidentification of very 'muscular' subjects as obese, our study's results might be suspect. It is likely that there were some subjects we identified as obese, who in fact were not obese. However, in general we tried to avoid recruiting subjects who had 'muscular' appearance, though of course it is not possible to know for sure any individual's body composition without an actual test of body density. We should also point out that although we were able to recruit subjects across the three classes of obesity, we only included 3 subjects who were in class III (BMI > 39.9), and thus our results cannot speak to the effect that might be found among the 'extremely'[29] obese. In any case, it is noteworthy that our percentage of obese subjects found to be positive for Ad-36 antibodies was similar to that found in Atkinson *et al*.'s study.[14]

It is possible, of course, that the study is flawed in some important respect that we have not identified. Such a flaw would deserve greater scrutiny in the face of our negative result. However, two strengths of the study argue against a significant methodological flaw. In the first place, the cases and controls were for the most part matched, minimizing the possible influence of demographic factors. Second, the validity of our methodology and findings are supported by the totality of our results. All of the variables we measured for associations with BMI mirror conventional associations.

We do not want to suggest that in light of our negative findings obesity is not related to pathogenic infections. A conversion of evidence of such associations, viral and bacterial, such as that provided by Dhurandhar's group[9,30] for the former, and Dart *et al*.[31] for the latter, may point to such a possibility. In this study, however, we found that, at least in this context, the possibility that Ad-36 has an important association with obesity is not strong. At the same time, we were able to identify demographic correlates of Ad-36 infection, which had previously not been reported.

Conflict of interest

The authors declare no conflict of interest.

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14. ABSTRACT
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Background. Several studies have shown a positive association between evidence of anti-Adenovirus 36 (Ad-36) antibodies (Ad-36 exposure) and (1) obesity and (2) serum cholesterol in animals. There is limited research on (1) demonstrating this association in humans and (2) transmission, presentation, and demographics of Ad-36 infection.
Design: (1) Body mass, (2) fasting serum cholesterol/triglyceride levels, (3) and demographic characteristics were compared between Ad-36 seropositive and seronegative groups.
Subjects: One hundred fifty obese and 150 lean active-duty military personnel were studied.
Measurements: Subjects completed a questionnaire regarding demographic/behavioral characteristics. Subject serum samples were tested by neutralization assay for presence of Ad-36 antibodies.
Results: Thirty-four percent of obese and 39% of lean subjects had Ad-36 exposure, an insignificant difference. Cholesterol/triglyceride levels were significantly higher among obese subjects than among lean, but there were no associations between cholesterol/triglyceride levels and Ad-36 exposure. Positive associations were found between Ad-36 exposure and age, race, and gender.
Conclusion: The study stands in contrast to previous work that has shown a positive relationship between Ad-36 exposure and (1) obesity and (2) levels of /cholesterol and triglycerides. There was no association in either case. Unanticipated relationships between Ad-36 exposure and age, race, and gender were found.

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Ad-36, adenoviruses, viral antibodies, obesity, military

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